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13. ABSTRACT ***(Maximum 200 Words)***

The object of this proposal is to understand the tumor suppressor function of maspin, a novel serine protease inhibitor, and to test directly maspin as a therapeutic agent for breast cancer. Transgenic and knockout mouse models will be employed to study the effects of gain and loss of maspin function on mouse mammary tumorigenesis and development. We hypothesize that overexpression of maspin should be protective against mammary tumorigenesis and metastasis, while loss of maspin will render mice more susceptible to tumor formation and metastasis.

The proposal is based upon our previous experiments, primarily performed in conventional cell culture models, demonstrating that maspin has tumor suppressor activity. Recently, we have established transgenic mice overexpressing maspin in the mammary gland, and generated maspin knockout mice. We have crossed maspin transgenic mice with a breast tumor WAP-Tag strain. Our data suggest that maspin functions directly as an inhibitor for angiogenisis and metastasis. We have also shown in this report the mechanism by which maspin inhibits normal mammary development and reduced tumor progression in bigenic mice. Continuation of these tasks in the next few years will help us understand the role of maspin in tumor metastasis and angiogenesis, and hopefully leading to the development of new therapies for the treatment of breast cancer.

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Ming Zhang, Ph.D., 4/10/2001

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Introduction

Breast cancer is the most common malignancy in western countries. Among women in the United States between the ages of 40 and 55 years, breast cancer is the leading cause of death (1). In the past, study of breast cancer has lagged behind other forms of cancer. Although the balance is being shifted, principally owing to women's awareness and increased funding, there are great gaps in our understanding of almost every aspect of this disease. Recently, The National Cancer Institute established a Breast Cancer Progress Review Group (BCPRG) to identify and prioritize scientific needs and opportunities that are critical to hasten progress against the disease. The proposal by this group has provided a guideline for individual breast cancer researcher to follow (Web site for this proposal: http://wwwosp.nci.nih.gov/planning/prg).

The object of this proposal is to understand the tumor suppressor function of maspin, a novel serine protease inhibitor, and to test directly maspin as a therapeutic agent for breast cancer. Transgenic and knockout mouse models will be employed to study the effects of gain and loss of maspin function on mouse mammary tumorigenesis and development. We hypothesize that overexpression of maspin should be protective against mammary tumorigenesis and metastasis, while loss of maspin will render mice more susceptible to tumor formation and metastasis. We will take advantage of the powerful tool of mouse genetics by crossing these mice with other well characterized mouse breast cancer models to test the tumor suppressor activity of maspin. Mammary tumorigenesis and normal mammary development will be studied using a variety of established techniques, including histopathology and whole mount analyses. Finally, we plan to deliver maspin locally as a drug to determine its therapeutic efficacy as an anti-tumor agent.

The following tasks were proposed for the first 12 month period of study.

Task 1. Analysis of the tumor suppressor activity of maspin by crossing WAP-maspin mice with WAP-Tag mice. Months 1-24. Total 100 mice will be used.

a. Generate four groups of mice by crossing WAP-maspin heterozygotes with WAP-Tag heterozygotes.
b. Continuous mating to activate the transgenes.
c. Collect mammary gland samples for histopathology and other studies.
d. Record tumor progression, and take tumor samples for histopathology.

Task 2. Tumor inhibition by overexpression of maspin in DMBA-treated mice.

a. Treat groups of transgenic and control mice with DMBA
b. Record tumor progression

Task 3. Tumor inhibition by local Evac implants of maspin. Months 6-20. Numbers of mice: 40

a. Prepare large quantity of recombinant GST-maspin protein and make maspin Evac pellets.

b. Implant pellets in the mammary fat pad near the site of mammary tumor developmen in WAP-Tag mice.
c. Biopsy of tumor samples, examine proliferation and apoptosis.

Task 4. Characterization of physiological functions of maspin by mean of targeted overexpression Months 6-24. Number of mice: 40

a. Do proliferation and apoptosis experiments using mammary gland biopsies from WAP-maspin mice to study effects on lobuloalveolar development.
b. Deliver maspin pellets to the mammary gland of normal 4-6 week old virgin mice anc post-lactation mice to study effects on ductal morphogenesis and involution.

Task 5. Effect of maspin gene disruption on mammary tumorigenesis and development. Month: 12-36. Number of mice: 200.

e. Gene knockout mice , if viable analyze mammary gland development.
f. Induce mammary tumorigenesis by DMBA in KO and control mice, observe tumo: progression and metastasis.

Body

Materials and methods

Animals

WAP-Tag mice were provided by Dr. Priscilla Furth as a collaboration. WAP-maspi transgenic mice were established in this laboratory. Maspin heterozygous mice were generated in thi: laboratory. Mouse tumors were provided by Dr. Dan Medina. TM40D mammary tumor implantatioı model was established in collaboration with Dr. Dan Medina. All animals were maintained within th PI's animal facility at Baylor.

Antibodies

Polyclonal anti-maspin antibody was made by Zymed, Inc. as a custom service. Anti-Taş antibody was purchased from Pharmingen. All secondary antibodies were purchased from Zymed, Inc.

Northern and Western analysis

RNAs and proteins were isolated from mammary glands from virgin, pregnant, lactatin; stages. Total RNAs were isolated using Gibco/BRL Trizol regent. For northern blot, roughly 20 u; RNA will be loaded each lane. For Western blot analysis, protein extracts were prepared by lysing th cells in RIPA buffer. Total 100 ug protein extract will be loaded for electrophoresis.

Immunohistochemical analysis

Mammary glands were removed under anesthesia from normal and transgenic females at different stages of development. Mammary tissues were fixed in 10% neutral formalin buffer and embedded in paraffin and sectioned at 5 μm. For maspin immunostaining, tissues were boiled in citrate buffer (Zymed, Inc.) for ten minutes for antigen retrival. The antibody was produced in rabbit against a fifteen amino acid peptide located in the reactive site loop of maspin (AbS4A) (2). The antibody was purified using an AbS4A sulfo-linked affinity column (Sulfolink kit, Pierce, IL). The sections were stained with the affinity purified maspin antibody at a dilution of 1:400, followed by a secondary goat anti-rabbit antibody staining, and the color was developed by Zymed's AEC chromogen kit. For specific peptide blocking, a concentration of 10 nM of AbS4A peptide was preincubated with antibody for thirty minutes at room temperature. For PCNA staining, a PCNA staining kit was purchased from Zymed (Zymed, Inc., CA) and slides were stained following the instruction of the kit. The anti-Tag antibody (pharminogen) will be diluted 1:400 times and used as instructed by Pharmingen. Anti-CD31 antibody for vessel density was purchased from Pharmingen.

Results and Discussion

Task 1. Analysis of the tumor suppressor activity of maspin by crossing WAP-maspin mice with WAP-Tag mice.

WAP-Tag transgenic mice (3) that are highly susceptible to mammary tumorigenesis were mated with WAP-maspin to generate four groups of mice: Tag +/maspin+ (25), Tag+/maspin-(15) Tag-/maspin+(16), Tag-/maspin-(15). They were mated continously to activate transgene expression Tumor progression data were recorded. Tumors first appeared in Tag+ mice about three months after first pregnancy, with 100% of them developed tumors by 6 months. Tumor growth was monitored by measurement with caliper biweekly and was followed until the tumor size reached 2.0 cm in diameter or tumor volume reached 10 % of body weight before the mice were sacrificed. At the moment, all mice were sacrificed and their samples harvested. Tumor free curves between biogenic and Tag+ were compared. No significant difference was observed for tumor incidence between these two groups. We conclude maspin overexpression does not prevent tumor initiation. Lung tissues were collected from tumor mice. Tumor metastasis to lung was evaluated by examining lung sections by H&E staining Our data showed tumor metastasis were reduced in bitransgenic mice (37.5%) comparing to the Tag+ mice (56.7%). It will be further examined by immunohistochemical analysis using an anti-Tag antibody. Current data indicate that overexpression of maspin in vivo can block tumor metastasis.

The mechanism of metastasis inhibition in biogenic mice was investigated. Tumor sections were analyzed for apoptosis rate and microvessel density by TUNEL assay and CD31 immunostaining.

Our data show that tumors from bigenic samples have increased apoptosis and decreased MVD, suggesting maspin may inhihit tumor progression by its anti-angiogenic property. The result was published in the Journal of Oncogene last year (7).

To further delineate maspin's role in angiogenesis inhibition, we carried out both endothelial cell motility assay and in vivo rat corneal assay. We demonstrated that maspin was an effective inhibitor of angiogenesis. In vitro it acted directly on cultured endothelial cells to stop their migration

towards bFGF and VEGF and to limit mitogenesis and tube formation. In vivo it blocked neovascularization in the rat cornea pocket model. Maspin derivatives mutated in the serpin reactive site lost their ability to inhibit the migration of fibroblasts, keratinocytes and breast cancer cells but were still able to block angiogenesis in vitro and in vivo. When maspin was delivered locally to human prostate tumor cells in a xenograft mouse model, it blocked tumor growth and dramatically reduced the density of tumor-associated microvessels (4).

Task 3. Tumor inhibition by local Evac implants of maspin. Months 6-20.

We have prepared maspin Evac to delivery maspin to mammary tumors. Instead of using WAP-Tag tumor mice, we started by using Balb/c mice and tumor tissues isolated from syngenic Balb/c mice since this model was well characterized by Dr. Dan Medina (5). All tumors and mice were provided by Dr. Daniel Medina as a collaboration. The study was carried out as followings. Tumor tissues were microscopically dissected into pieces for implanting to the fat pad of 8 week old Balb/c female mice. They were allowed to grow for two weeks inside mammary gland. Maspin slow release Evacs were surgically implanted beside the tumor sites and the incisions were closed. Two weeks later, tumors were excised and their sizes were measured. Initial experiment showed maspin treated tumors have reduced size but due to the large variation of tumor size and small scale of first experiment, the difference was not proved to be statistically significant. In view of recent data from biogenic experiment, it is also possible that maspin delivered may not be sufficient to inhibit tumor growth. Another possibility is that the delivery efficiency needs to be optimized. To further examine maspin's inhibitory effect, we decided to consolidate task2 and 3 and designed a new mammary tumor model. This model involved the implantation of mammary tumor cells orthotopically to mammary gland and tumors were allowed to grow within the gland and become invasive and metastatic to other organs. Here we demonstrated that TM40D cells in implanted mammary glands were highly invasive. Overall, 75% rate of invasion and metastasis was observed in this model. However, both primary tumor growth and metastasis were significantly blocked in TM40D cells transfected with maspin by plasmid or retrovirus infection. Maspin transfected tumors tended to have tumor encapsulation and less necrosis, which were associated with better prognosis and lower invasiveness. Thus, maspin itself indeed can block primary tumor growth as well as invasion and metastasis. This study was recently completed and a manuscript is in preparation (see reportable outcomes).

Task 4. Characterization of physiological functions of maspin by mean of targeted overexpression. Months 6-24.

We have found that overexpression of maspin at midpregnancy inhibits mouse mammary gland development (6). One hypothesis is that maspin may be involved not only in extracellular matrix remodeling but also as a regulator for cell proliferation. On the other hand, over-expression of maspin may inhibit mammary gland development by inducing extensive apoptosis. To understand whether the induction of transgene expression caused any changes of alveolar cells in proliferation and apoptosis, PCNA staining and TUNEL assay were carried out with midpregnant mammary samples. Our data indicate that the maspin transgene does not change the proliferation rate, but increases significantly the apoptosis of alveolar cells. The increase was sustained even to the late stage of pregnancy when normal gland had little apoptosis. Since milk protein genes can function as differentiation markers for the mammary gland, we compared their expression patterns in transgenic and wildtype control mice. Western blot analysis showed that WAP and β-casein were highly expressed in wild-type mammary

gland at day 19 pregnancy and throughout the three stages of lactation. However, neither WAP nor β-casein was detected in our assay at day 19 pregnancy in transgenic glands as compared to control. The levels of both milk proteins were present in lactating day 1 transgenic glands, but at a reduced level. Following three days of lactation, WAP and β-casein levels in the transgenic mice increased to that of control. The decreased expression of milk genes could arise from the reduced number of alveoli, as well as lower expression by each alveolar epithelial cell.

In summary, maspin transgene expression resulted in a decrease in both the number of lobular-alveolar structures and the size of each alveolar unit during pregnancy and early lactation, and this effect is due to the increased rate of apoptosis. The result was published in the journal of Developmental Biology (6).

Task 5. Effect of maspin gene disruption on mammary tumorigenesis and development.

We have generated maspin knockout mice in order to examine maspin's role in mammary tumor progression. Unfortunately, the homozygotes are lethal in embryo development. That limited our ability to study the mammary development at late stages. Utilizing maspin heterozygote mice, the loss of maspin on tumorigenesis mediated by the carcinogen DMBA (Dimethylbenz[a]anthrazene) which induces an oncogenic process in the mammary gland, was investigated. At five weeks of age pituitary isografts were implanted to stimulate lobulalveolar development of the mammary gland. Groups of experimental mice (15 heterozygotes) and controls (15 mice) were given DMBA at 0.5mg/ml at 9 and 10 weeks of age to stimulate mammary tumorigenesis. Surprisingly, the maspin +/- mice showed a delay in tumor incidence and a decrease in tumor burden (Figure 1). However, since the maspin +/- mice

display an ovarian phenotype (data not shown), the possibility exists that the delay in tumor formation may be due to late ovulatory failure. Indeed, the response of the mammary gland to the pituitary isograft and later proliferation is dependent upon hormones produced by the ovary. Thus, any compromise in the ovary would result in a decrease in proliferation and tumorigenesis. The likelihood also exists that this response may be due to a subtle inherent mammary gland phenotype in the maspin +/- mice; however, these mice were shown to respond to the pituitary isograft as compared to wild type mice as determined by whole mount analysis. Under the advice of Dr. Daniel Medina, an expert in mammary carcinogenesis at Baylor College of Medicine, we are currently repeating this experiment utilizing mice, which have been ovarectomized, and inducing proliferation by placing permeable tubing containing estrogen + progesterone under the skin. These tubes will be replaced every six weeks until the end of the experiment.

We are also investigating whether loss of one copy of maspin in the heterozygotes causes any defect in mammary gland development by histological analysis.

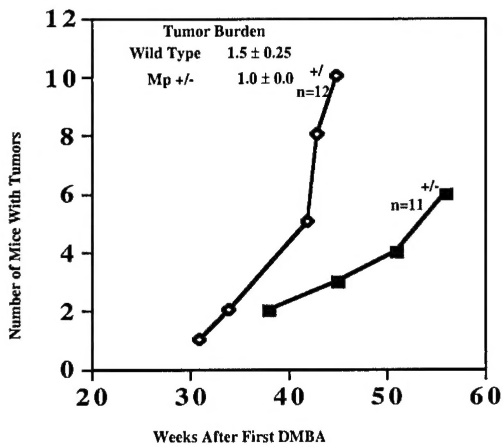

Figure 1: Tumor Incidence

Key research accomplishments

- ❖ Overexpression of maspin in transgenic mice inhibits mammary gland development.
- ❖ Targeted overexpression of maspin reduced the rate of tumor progression and metastasis.
- ❖ Maspin is a potent angiogenesis inhibitor.
- ❖ Maspin knockout mice are homozygous lethal. Heterozygous mice may display phenotypes after being challenged by chemical carcinogen.
- ❖ Overexpression of maspin in TM40D implantation model blocks tumor growth, invasion and metastasis.

Reportable Outcomes

Three papers were published with the support of this grant proposal. One manuscript on maspin overexpression study (task 2 and 3) is in preparation. Task 5 is still in progress.

1. Zhang, M., Magit, D., Botteri, F., Shi, Y., He, K., Li, M., Furth, P., Sager, R. Li, M., Furth, P., Sager, R. Maspin plays an important role in mammary gland development. **Developmental Biology**, 215: 278-287, 1999.
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4. Heidi Y Shi, Weiguo Zhang, Rong Liang, Shaji Abraham, Francis S. Kittrell, Dan Medina, Ming Zhang. Blocking tumor growth, invasion and metastasis by maspin in a syngeneic breast cancer model . In preparation.

Conclusion

All tasks proposed in the grant were carried out in the last three years of proposal. We have obtained very informative data suggesting maspin functions directly as a metstasis inhibitor. We have uncovered the mechanism by which maspin inhibits normal mammary development during pregnancy in WAP-maspin transgenic mice, and we are testing maspin against tumor progression in mice. We have also demonstrated that maspin inhibits angiogensis. Continuation of these tasks in the next few years will help us understand the role of maspin in tumor metastasis and angiogenesis, and hopefully leading to the development of new therapies for the treatment of breast cancer.

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7. Zhang, M., Shi, Y., Magit, D., Furth, PA., Sager, R. Reduced mammary tumor progression in WAP-TAg /WAP-maspin bitransgenic mice. **Oncogene**